

FORM PTO-1390 (Modified)
(REV 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

A34537-PCT-USA

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/890779

INTERNATIONAL APPLICATION NO.
PCT/EP00/01524INTERNATIONAL FILING DATE
09 February 2000PRIORITY DATE CLAIMED
09 February 1999

TITLE OF INVENTION

A METHOD FOR INHIBITING THE EXPRESSION OF TARGET GENES

APPLICANT(S) FOR DO/EO/US

WERR, Wolfgang

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☒ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Form PCT/IB/304; Form PCT/IB308; Form PCT/IB332; Form PCT/RO/101; a postcard and a check in the amount of \$860.

EXPRESSMAIL LABEL NO.:

Date of Deposit: August 6, 2001

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 09/890779	INTERNATIONAL APPLICATION NO. PCT/EP00/01524	ATTORNEY'S DOCKET NUMBER A34537-PCT-USA
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24. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- | | | |
|-------------------------------------|---|------------------|
| <input type="checkbox"/> | Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO | \$1000.00 |
| <input checked="" type="checkbox"/> | International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO | \$860.00 |
| <input type="checkbox"/> | International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO | \$710.00 |
| <input type="checkbox"/> | International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) | \$690.00 |
| <input type="checkbox"/> | International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) | \$100.00 |

ENTER APPROPRIATE BASIC FEE AMOUNT =

CALCULATIONS PTO USE ONLY

\$860.00

Surcharge of **\$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).

\$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	16 - 20 =	0	x \$18.00	\$0.00	
Independent claims	1 - 3 =	0	x \$80.00	\$0.00	
Multiple Dependent Claims (check if applicable).			<input type="checkbox"/>	\$0.00	

Multiple Dependent Claims (check if applicable).

TOTAL OF ABOVE CALCULATIONS =

\$860.00

- ☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.

\$0.00

SUBTOTAL =

\$860.00

Processing fee of **\$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).

\$0.00

TOTAL NATIONAL FEE =

\$860.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).

\$0.00

TOTAL FEES ENCLOSED =

\$860.00

**Amount to be:
refunded**

§

charged

\$

- a. ☒ A check in the amount of \$860.00 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4377 A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Janet M. MacLeod
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Janet M. MacLeod
SIGNATURE

Janet M. MacLeod

NAME _____

35,263

REGISTRATION NUMBER

6 August 2001

DATE _____

JC05 Rec'd PCT/PTO 09/890779
0.6 AUG 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: WERR, Wolfgang
Serial No.: to be assigned
Filed : 9 February 2000
For : A METHOD FOR INHIBITING THE
EXPRESSION OF TARGET GENES IN PLANTS

PRELIMINARY AMENDMENTEXPRESS MAIL NO. EK839853593 US**BOX PCT**

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application, please amend the
application as follows:

IN THE SPECIFICATION:

At page 1, after the title, please insert the following paragraph:

--This application is a 371 of international application PCT/EP00/01524
which was published in English.--

IN THE ABSTRACT:

After the claims, please insert the attached single page containing the abstract.

IN THE CLAIMS:

Please amend the claims as follows:

4. (Amended) A chimeric DNA construct according to claim 1, wherein said plant-specific sequence codes for at least a DNA-binding domain of a plant transcription factor.

6. (Amended) A chimeric DNA construct according to claim 1, wherein said plant-specific sequence activates transcription by interacting with a DNA-binding protein.

7. (Amended) A chimeric DNA construct according to claim 1, further comprising a sequence that encodes at least the hormone-binding domain of a steroid hormone receptor, said sequence being in frame with the fusion construct consisting of at least one repressor sequence in transcriptional fusion with at least one plant-specific sequence that codes for a protein or fragment thereof that activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein; whereby the nuclear localization of the chimeric protein, that is the product of the translation of said fusion construct is dependent on the application of said steroid hormone or analogues thereof.

9. (Amended) A chimeric DNA construct according to claim 1, wherein the elements allowing the transcription of said repressor sequence comprise a constitutive promoter.

10. (Amended) A host cell transformed with a DNA construct according to claim 1.

11. (Amended) A transgenic plant or parts thereof, said plant being transformed with a DNA construct according to claim 1, or deriving from said plant.

12. (Amended) A method of obtaining a transgenic plant, wherein a DNA construct according to claim 1 is transferred and expressed in a plant cell and said cell is cultured under conditions for regenerating a whole transgenic plant.

13. (Amended) Use of a chimeric DNA construct according to claim 1 for inhibiting the expression of a target gene in the genome of a plant.

14. (Amended) A method for determining the function of a transcription factor in plants, comprising the steps of:

- i) fusing a sequence encoding said transcription factor to a repressor sequence to form a DNA construct as defined in claim 4;
- ii) transforming plant cells with said DNA construct;
- iii) culturing the plants obtained from the transformed cells and observing a phenocopy of a mutation correlated with the loss of expression of genes controlled by said transcription factor.

15. (Amended) A method for identifying new genes in plants, comprising the steps of:

- i) obtaining transgenic plants transformed with a chimeric DNA construct according to claim 1;
- ii) comparing the RNA population from said transgenic plants with the RNA population of a plant that has not been transformed with said chimeric DNA construct, by amplifying the RNAs repressed by expression of the chimeric DNA

construct, identified as genes inactive in said transgenic plants obtained in step i) but active in the plant that has not been transformed with a chimeric DNA construct.

16. (Amended) A method for identifying new genes in plants, comprising the steps of:

i) obtaining transgenic plants transformed with a chimeric DNA construct according to claim 7, comprising a sequence that encodes at least the hormone-binding domain of a steroid hormone receptor;

ii) submitting said transgenic plants to an induction by means of a steroid hormone or analogues thereof, whereby a phenocopy is created due the loss of expression of target genes;

iii) comparing the RNA populations from said transgenic plants before and shortly after induction, by amplifying the RNAs repressed by expression of the chimeric DNA construct, identified as genes active before but inactive after induction.

ABSTRACT

This invention concerns a method for inhibiting the expression of target genes in plants wherein a chimeric DNA construct comprising at least one repressor sequence in transcriptional fusion with at least one plant-specific sequence that codes for a protein or fragment thereof that bind to DNA or that activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein.

REMARKS

The claims have been amended to remove multiple dependencies. The specification has been amended to indicate that the international application was published in English, and to include an abstract.

Favorable consideration of all pending claims is earnestly solicited.

Attached hereto as **APPENDIX A** is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "**VERSION WITH MARKINGS TO SHOW CHANGES MADE.**"

Respectfully submitted,

Dated: August 6, 2001


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APPENDIX A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

At page 1, after the title, the following paragraph was inserted:

--This application is a 371 of international application PCT/EP00/01524

which was published in English.--

IN THE ABSTRACT:

After the claims, the attached single page containing the abstract was inserted.

IN THE CLAIMS:

Claims 4, 6-7, and 9-16 have been amended as follows:

4. (Amended) A chimeric DNA construct according to [any of claims] claim 1 [to 3], wherein said plant-specific sequence codes for at least a DNA-binding domain of a plant transcription factor.

6. (Amended) A chimeric DNA construct according to [any of claims] claim 1 [to 3], wherein said plant-specific sequence activates transcription by interacting with a DNA-binding protein.

7. (Amended) A chimeric DNA construct according to [any of claims] claim 1 [to 6], further comprising a sequence that encodes at least the hormone-binding domain of a steroid hormone receptor, said sequence being in frame with the fusion construct consisting of at least one repressor sequence in transcriptional fusion with at least one plant-specific sequence that codes for a protein or fragment thereof that activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein;

whereby the nuclear localization of the chimeric protein, that is the product of the translation of said fusion construct is dependent on the application of said steroid hormone or analogues thereof.

9. (Amended) A chimeric DNA construct according to [any of claims] claim 1 [to 8], wherein the elements allowing the transcription of said repressor sequence comprise a constitutive promoter.

10. (Amended) A host cell transformed with a DNA construct according to [any of] claim 1 [to 9].

11. (Amended) A transgenic plant or parts thereof, said plant being transformed with a DNA construct according to [any of claims] claim 1 [to 9], or deriving from [a] said plant [initially transformed with a DNA construct according to any of claims 1 to 9].

12. (Amended) A method of obtaining a transgenic plant [according to any of claims 10 or 11], wherein a DNA construct according to [any of claims] claim 1 [to 9] is transferred and expressed in a plant cell and said cell is cultured under conditions for regenerating a whole transgenic plant.

13. (Amended) Use of a chimeric DNA construct according to [any of claims] claim 1 [to 9] for inhibiting the expression of a target gene in the genome of a plant [, the transcription of which is activated by a protein encoded by a plant-specific sequence as defined in any of claims 1 to 9].

14. (Amended) A method for determining the function of a transcription factor in plants, comprising the steps of:

i) fusing a sequence encoding said transcription factor to a repressor sequence to form a DNA construct as defined in [any of claims] claim 4 [to 9];

ii) transforming plant cells with said DNA construct;

iii) culturing the plants obtained from the transformed cells and observing a phenocopy of a mutation correlated with the loss of expression of genes controlled by said transcription factor.

15. (Amended) A method for identifying new genes in plants, comprising the steps of:

i) obtaining transgenic plants transformed with a chimeric DNA construct according to [any of claims] claim 1 [to 6];

ii) comparing the RNA population from said transgenic plants with the RNA population of a plant that has not been transformed with [a] said chimeric DNA construct [according to any of claims 1 to 6], by amplifying the RNAs repressed by expression of the chimeric DNA construct, identified as genes inactive in said transgenic plants obtained in step i) but active in the plant that has not been transformed with a chimeric DNA construct.

16. (Amended) A method for identifying new genes in plants, comprising the steps of:

i) obtaining transgenic plants transformed with a chimeric DNA construct according to [claims] claim 7 [or 8], comprising a sequence that encodes at least the hormone-binding domain of a steroid hormone receptor [, and/or according to claim 9, comprising an inducible promoter];

ii) submitting said transgenic plants to an induction by means of a steroid hormone or analogues thereof [and/or promoter inducer], whereby a phenocopy is created due the loss of expression of target genes;

iii) comparing the RNA populations from said transgenic plants before

and shortly after induction, by amplifying the RNAs repressed by expression of the chimeric DNA construct, identified as genes active before but inactive after induction.

ABSTRACT

This invention concerns a method for inhibiting the expression of target genes in plants wherein a chimeric DNA construct comprising at least one repressor sequence in transcriptional fusion with at least one plant-specific sequence that codes for a protein or fragment thereof that bind to DNA or that activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein.

09/890779

A method for inhibiting the expression of target genes in plants

The present invention relates to a method for inhibiting the expression of target genes in plants and to DNA constructs used to implement this method.

Plant transcription factors mostly belong to gene families containing numerous members. An example how large such families can grow is the *myb*-family comprised of at least 97 genes in *Arabidopsis*. Also homeobox and MADS box genes are found in multiple copies comprising several subfamilies in all plant species analyzed so far. In genetic screens for loss of function alleles only few of these genes have been associated with a mutant phenotype. One explanation for numerous silent family members may be genetic redundancy; each gene specific contributions may be subtle or dependent on special conditions. This fraction of silent genes in plant as others genomes provides a major challenge in molecular biology because these are foreseen to oppose a functional analysis also in reverse genetic approaches.

In the transformation of plants, to alter the structure and/or the function of whole plants, it may be desirable to block the expression of genes. This may be effected by altering either the sequence of the gene itself or of the transcriptional factor that controls its transcription. For that purpose the antisense technology, as described for example in the US patent 4,943,674, may be used. Site-directed mutagenesis may alternatively be used. It has for example been proposed to use in vitro altered transcriptional activators encoding proteins that bind to one or more components of the transcription complex in such a way that they competitively inhibit expression of target gene or genes in plants (EP 0 475 584). Co-suppression system may also be used to block expression of specific genes (Flavell R.B., 1994).

However these technologies require an accurate knowledge of the structure of the gene or of the transcriptional factor (namely of the DNA binding and transactivator domains) to allow the antisense sequence to specifically hybridize with the gene sequence, or to allow the mutation to only

affect some properties of the protein (DNA binding or activator domain) respectively.

Other limitations of these methods are well known by one skilled in the art : in the case of redundancy in genes and/or functions, other related
5 genes, non altered by these targeted technologies, conceal the expression of the target gene. As a result, the observed phenotype is wild-type instead of mutant as expected. Furthermore these techniques make the isolation of new genes, or the determination of new functions difficult, sometimes impossible.

The authors of the present invention now propose an alternative
10 approach for blocking the expression of genes in plants which meets the above requirements and presents other advantages. The method of the invention distinguishes from previous technologies in that this is, as further described, a dominant approach with high penetrance. This method is independent from any DNA sequence homology with the targeted gene, which is crucial for antisense
15 and cosuppression approaches, and moreover circumvents the problem of redundancy in genes and/or functions.

The method of the invention involves the use of a chimeric DNA construct comprising at least one repressor sequence in transcriptional fusion with at least one plant-specific sequence that codes for a protein or fragment
20 thereof that bind to DNA or that activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein ;

said repressor sequence being operably linked to elements allowing the transcription of said fused sequences.

The term "plant-specific sequence" means a sequence that
25 originates from plants or has been modified (or shown) to function in plants, i.e. to actually bind to DNA in plants or to activate transcription either by binding to DNA itself or by interacting with a DNA-binding protein.

It is understood that the repressor sequence of said chimeric DNA construct preferably does not contain a sequence that codes for a second other
30 DNA-binding domain, so that no undesirable interaction could occur.

In a preferred embodiment, said repressor sequence is at least the repressor domain of the *Drosophila engrailed* gene (*eng*).

The product of the *Drosophila engrailed (eng)* locus was shown to be involved in the regulatory interactions that govern early embryogenesis (Poole et al, 1985). Eng is a homeodomain-containing transcription factor that is required for cell fate specification through out development of the fly. A pioneering experiment was performed in *Drosophila* where the DNA-binding homeodomain of the *engrailed (eng)* gene was replaced by its *Fushi tarazu (Ftz)* counterpart, and expression of the chimeric gene controlled by heat shock promoter. After induction, the sequence specificity of the *Ftz* homeodomain directed the strong transcriptional *eng* repressor function to *Ftz* target genes resulting in phenocopies of *Ftz* loss of function alleles in transgenic progeny (John et al., 1995). Thus, the Eng protein was shown to be an active repressor in *Drosophila* (John et al, 1995) and cultured animal cells (Han & Manley, 1993). In 1996, Conlon et al use an approach in which the DNA-binding domain of a transcription activator is fused to the *engrailed* repressor domain to assist in the analysis of *Xenopus* and *Zebrafish* transcription factors (Conlon et al, 1996). But no stable transformation has been carried out until now in plants, in order to obtain transgenic plants with heritable mutant phenotype.

According to the present invention, the term "repression domain of the *Drosophila engrailed* gene (*eng*)" means a fragment of the *Drosophila engrailed* gene or of a derivative sequence thereof, said fragment comprising a nucleotide sequence that encodes a polypeptide interfering with the general transcription machinery and transcriptional activators. Said fragment advantageously comprises a minimal repression sequence coding for a polypeptide of 55 residues (Poole et al., 1985, Han K. et al., 1993). The whole *engrailed* gene may also be used but most preferably without its homeodomain.

"A derivative sequence" is understood as meaning a sequence which differs from the sequence of the *Drosophila engrailed* gene by mutation, insertion, deletion or substitution of one or more bases, or by the degeneracy of the genetic code so long as it codes for a polypeptide which is substantially the same as the Engrailed product.

Engrailed related domains, which share the same repression activity, may also be used, such as those described in Smith et al. (1996).

In another embodiment, the repressor sequence that is used may be a sequence that codes for at least the Kruppel-associated box-A (KRAB-A) domain of zinc finger proteins (Witzgall et al., 1994) for at least the RE-1-silencing transcription factor (REST) (Thiel et al., 1998), or for at least the BTB (for Broad-complex Tramtrac and Bric) domain, also known as POZ-domain (Ahmad et al., 1998 ; Huynh & Bardwell, 1998).

Chimeric DNA constructs further comprising corepressor sequences associated with said repressor sequence are also encompassed. Among the corepressors of interest, one may cite KAP-1 (Friedman et al., 1996), groucho (Tolkunova et al., 1998), or KOX-1 (Moosmann et al., 1997), or N-Cor and SMRT (Huynh and Bardwell, 1998).

In one embodiment, said repressor sequence is fused to a plant-specific sequence that encodes a DNA-binding protein domain. The term "DNA binding protein domain" refers to the whole protein that contains such a DNA-binding domain but also to parts of it independently of their ability to bind to DNA.

In a preferred embodiment, a plant transcription factor, or a DNA-binding fragment thereof, is used as the DNA-binding domain containing protein. A complete transcription factor is preferably used, as it preserves interactions between proteins involved in regulation of transcription.

In an alternative embodiment, the repressor sequence is fused to a sequence that codes for a protein or parts thereof that activates transcription by binding to a DNA-binding protein such as a transcription factor. Such proteins may either increase the affinity of DNA-binding proteins to their target sites or mediate signalling to the transcriptional initiation complex and are generally called coactivators.

In the preferred embodiment wherein the DNA-binding domain containing protein is a plant transcription factor, said transcription factor may preferably be selected from the group consisting of :

- STM ("Shootmeristemless"), member of the Knotted class of homeodomain proteins, which is an essential gene for development and function of the shoot apical meristem ;

- AP3 ("APETALA3"), member of the MADS box proteins, which is a floral organ identity B function gene essential for the development of petals and stamen. AP3 protein is known to form heterodimers with the PISTILLATA (PI) gene product.

STM and AP3 encode plant transcription factors with well-characterized loss of function alleles. Both genes are not only members of different classes of plant transcription factors but deficiencies in both genes cause phenotypic effects at different developmental stages, early in the vegetative phase or rather late in the floral development.

- ZmHox - member of the homeobox proteins, more particularly ZmHox 1a/1b and 2a/2b, which are expressed in maize meristems and proliferating cells from the early embryo to late reproductive organs. This expression pattern suggests a contribution to plant growth and morphogenesis;

- Ms-41-A and Zm-41-A. These two factors are associated with male fertility. Recent results ascertain that Ms41-A protein from *Arabidopsis* is a transcription factor belonging the family recently described as ARF1 family for Auxin Response Factor 1 (Ulmasov et al., 1997). Ms41-A analogues may also be used, such as those described in WO 97/23618 or the *Arabidopsis* gene Monopteros which encodes a transcription factor mediating embryo axis formation and vascular development (Hardtke et al., 1998), and the *Arabidopsis* gene ETTIN involved in floral development (Sessions, 1997).

The plants transformed with a chimeric DNA construct of the invention comprising such Ms-41-A factor are expected to be male sterile.

In one embodiment, the chimeric DNA construct of the invention may further comprise a sequence that encodes at least the hormone-binding domain of a steroid hormone receptor, said sequence being in frame with the fusion construct consisting of at least one repressor sequence in transcriptional fusion with at least one plant-specific sequence that codes for a protein or

fragment thereof that activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein ;

whereby the nuclear localization of the chimeric protein, that is the product of the translation of said fusion construct is dependent on the application of said steroid hormone or analogues thereof.

The steroid hormone receptor that is used may be for example the glycocorticoid receptor (GR). In that case, dexamethasone may be used to induce the nuclear translocation of the chimeric protein.

Optionally, we can use the amino acid sequences which, when included in a protein, function to promote transport of the protein to the nucleus are known in the art and are termed nuclear localization signals (NLS).

The repression domain of the *Drosophila engrailed* gene (*eng*) is operably linked to elements allowing its expression. Such elements may more particularly comprise a promoter and polyadenylation signals.

In one embodiment, the promoter that is used is a constitutive promoter.

In another embodiment, the promoter that is used is a tissue-specific promoter or a developmentally regulated promoter. This allows a conditional loss of functions that may be desired, for example to design new plant varieties.

In still other embodiments, the promoter that is used is an inducible promoter.

The polyadenylation that can be used may be for example the 35S polyA terminator of cauliflower mosaic virus (CaMV), as disclosed in Franck et al. (1980) and NOS polyA terminator, that corresponds to the non coding 3' region of nopaline synthase of Ti plasmid of *Agrobacterium tumefaciens* (Depicker et al., 1982).

Among the preferred promoters that can be used, one can cite for example :

a) constitutive promoters :

- 35S promoter, or advantageously the double 35S constitutive promoter of CaMV as described in Kay et al. (1987) ;

- rice actin promoter followed by the rice actin intron (PAR-IAR) included in plasmid pAct1-F4 as described in Mc Elroy et al. (1991);
- the constitutive promoter EF-1 α of the gene encoding for plant elongation factor described in WO 90/02172 or in Axelos et al. (1989);
- 5 - chimeric superpromoter PSP (Ni et al., 1995) constituted by the fusion of a triple repeat of the transcriptional activity element from the promoter of the gene of *Agrobacterium tumefaciens* octopin syntase, the transcriptional activating element of the promoter of the gene *Agrobacterium tumefaciens* mannopin synthase ; and
- 10 - ubiquitin promoter from sunflower (Binet et al., 1991).

b) specific promoters :

• *seed-specific promoters* :

- PCRU promoter of radish cruciferin gene allowing the
- 15 expression specifically in seeds, as described in Depigny-This et al. (1992) ;
- HMWG promoter (High Molecular Weight Glutenin) from barley (Anderson et al., 1989) ;
- the promoter of maize γ zein (P γ zein) included in p γ 63 plasmid in Reina et al. (1990) allowing the expression in albumen of maize
- 20 seeds ;
- PGEA1 and PGEA6 promoters corresponding to the non coding 5' region of the genes GEA1 and GEA6, expressed in the grains in *Arabidopsis thaliana* (Gaubier et al., 1993) ; and
- β -phaseolin promoter (Riggs et al., 1989).

25 • *specific promoters which drive expression in particular plant tissues which are involved in the control of fertility* :

Among the promoters of interest one may cite the Brassicaceae A3 or A9 promoter described in WO 92 11379, the A6 promoter described in WO 93 02197, or TA29, TA26, TA13 promoters

30 described in WO 89 10396 ;

- the Ms41-A anther-specific promoter described in WO 97/23618, which may also be used in male sterility systems ;

- a dehiscence-zone specific promoter such as the one described in EP 692 030.

c) inducible promoters :

- a promoter inducible in stress conditions, for example heat shock, wound or interaction with pathogens (Kuhlemeier et al., 1987, WO 94/21793) ; and
- an ethanol-inducible promoter (Salter et al., 1998) ; and
- the PR1a promoter inducible by salicylic acid for example (US 5,689,044).

The DNA construct of the invention is advantageously inserted in a vector, e.g. a plasmid, for use in plant cell transformation.

The transformation of plant cells may be effected by transferring the above vectors in protoplasts, in particular after incubating those protoplasts in a solution of polyethyleneglycol (PEG) in the presence of divalent cations (Ca^{2+}) as described in Krens et al. (1982).

The transformation of plant cells may also be effected by electroporation as described in Fromm et al. (1986).

A gene gun may also be used allowing the projection of metal particles coated with a DNA construct of the invention, whereby genes are delivered into cell nucleus (Sanford et al., 1988).

Another method for transforming plant cells is cytoplasmic or nuclear micro-injection.

In a preferred embodiment, plant cells are transformed with a DNA construct of the invention, by means of a host cell infecting said plant cells. A further subject of the present invention is thus a host cell transformed with a chimeric DNA construct as previously described. Advantageously, the above host cell is *Agrobacterium tumefaciens*, as used in particular in the methods of Bevan et al. (1984) and An (1986), or *Agrobacterium rhizogenes*, in particular as used in the method of Jouanin et al. (1987).

Plant cell transformation is preferably effected by transferring the *Agrobacterium tumefaciens* T region of an extra-chromosomal circular plasmid that induces tumors (Ti) e.g by using a binary system.

For that purpose, two vectors are constructed. In one of these
5 vectors, the T-DNA region is removed by deletion except for the right and left borders, a marker gene being inserted between the two borders to allow the selection in plant cells. The other partner of the binary system is a helper Ti plasmid which is a modified plasmid that has no T-DNA longer but still contains the virulence genes *vir*, necessary for transforming a plant cell. This plasmid is
10 maintained in *Agrobacterium*.

The present invention also provides a transgenic plant or parts thereof, said plant being transformed with a DNA construct of the invention, or deriving from a plant initially transformed with a DNA construct of the invention. Such transgenic plants exhibit heritable phenotypes.

15 The term "deriving" refers to plants of the following generations, as long as the parent plant is fertile.

The term "parts" of transgenic plants refer in particular to leaves, fruits, seeds, roots or cells that have been genetically transformed.

The preferred plants that are used for transformation may be for
20 example selected from the group consisting of *Arabidopsis thaliana*, rice, tobacco, maize, Brassica, wheat, tomato and flowers (*Petunia*, rose, carnation).

The present invention thus provides a method for obtaining a transgenic plant, wherein a DNA construct of the invention is transferred and
25 expressed in a plant cell and said cell is cultured under conditions for regenerating a whole transgenic plant.

The conditions for regenerating a whole plant from a plant cell are well-known by one skilled in the art.

30 A chimeric DNA construct of the invention may be used for inhibiting the expression of a target gene in the genome of a plant, the transcription of which involves a DNA-binding protein domain as previously defined.

Inhibiting the expression of a target gene in the genome of a plant may be desired in many purposes. Many transcription factors known by the man skilled in the art play a role in the control of metabolic pathways (starch, lipids, amino acids...) or are involved in the plant development, or in the plant sensibility to pathogen. For example, blocking a gene whose expression is necessary for pollen or another formation (e.g. the Ms-41-A transcription factor, as above described) produces male sterility. Blocking the gene controlled by the AP-3 transcription factor also leads to male sterility. As another example, blocking the gene which codes for the enzyme which catalyses the conversion of sugars to starch can be used to produced sweet corn (see EP 475 584).

It is also possible to obtain transgenic plants with enriched content in lysine by using, according to the invention, the opaque 2 transcription factor (Schmidt et al., 1990), involved in the control of the expression of certain zeins. One could further use Myb-related transcription factors involved in the control of anthocyanin biosynthesis in flowers (Martin et al., 1991 ; Matin, 1997), to modify their colour. Use of other members of Myb-related transcription factors playing a role in the regulation of phenylpropanoid and lignin biosynthesis (Tamagnone et al., 1998) could also be interesting. Some others could be involved in cellular development and senescence.

The chimeric construct, fusion of the repressor domain to the plant transcription factor or part of it, can be advantageously used to identify essential protein-protein interaction domains and interacting protein partners in planta in transgenic plants.

In a preferred embodiment, expression of a chimeric protein comprising the repressor domain in fusion to a plant transcription factor is known to cause a dominant phenocopy. By the deletion of increasing parts of plant sequences encoding the transcription factor and their repression in fusion to the repressor domain in transgenic plants, essential protein domains can be identified. These are DNA-binding domains but also others, like protein-protein interaction domains, if the transcription factor is part of multi-component complex. The method according to the invention is therefore also suitable to study protein-protein interactions in planta.

The chimeric DNA construct of the invention can be used to produce phenocopies of loss of function mutants in genes involved in transcriptional control, by reversing the biological function from activation to repression and by providing chimeric fusion proteins in excess over the endogenous function.

Phenocopy is an artificial (transgenic) situation mimicking a mutant phenotype. The term phenocopy is used by Smith et al. (1996), referring to the original description of John et al. (1995), describing a Ftz loss of function phenotype mimicked by the Eng Ftz chimeric protein.

The phenocopy caused by the expression of the chimeric fusion protein allows to associate a biological function to a given transcription factor or gene involved in transcriptional control.

A chimeric DNA construct of the invention may more particularly be used in a method for determining the function of a transcription factor in plants, comprising the steps of :

- i) fusing a sequence encoding said transcription factor to a repressor sequence to form a DNA construct as previously defined;
- ii) transforming plant cells with said DNA construct;
- iii) culturing the plants obtained from the transformed cells and observing a phenocopy of a mutation correlated with the loss of expression of genes controlled by said transcription factor.

The chimeric DNA construct of the invention allows the production of a cDNA library, said library being useful to isolate new genes controlled by the transcriptional activator as above described.

The present invention thus encompasses a method for identifying new genes in plants comprising the steps of :

- i) obtaining transgenic plants transformed with a chimeric DNA construct of the invention ;
- ii) comparing the RNA population from said transgenic plants with the RNA population of a plant that has not been transformed with a chimeric DNA construct of the invention, by amplifying (for example by Polymerase

Chain Reaction in a differential display approach (Liang et al., 1993) the RNAs repressed by expression of the chimeric DNA construct, identified as genes inactive in said transgenic plants but active in the plant that has not been transformed with a chimeric DNA construct of the invention.

5 The plants to be compared should genotypically be as identical as possible. Most preferably, the plants that have not been transformed with a chimeric DNA construct of the invention are transgenic plants transformed with a blank vector.

10 In a preferred embodiment, the method for identifying new genes in plants comprises the steps of :

 i) obtaining transgenic plants transformed with a chimeric DNA construct comprising a sequence that encodes at least the hormone-binding domain of a steroid hormone receptor and/or comprising an inducible promoter;

15 ii) submitting said transgenic plants to an induction by means of a steroid hormone or analogues thereof and/or promoter inducer, whereby a phenocopy is created due to the loss of expression of target genes;

 iii) comparing the RNA populations from said transgenic plants before and shortly after induction, by amplifying (for example by Polymerase Chain Reaction in a differential display approach (Liang et al., 1993) the RNAs repressed by expression of the chimeric DNA construct, identified as genes active before but inactive after induction.

20 In a preferred embodiment, a differential display approach associated with the AFLP technique described by Vos et al. (1995) or the PCR-Select System (Clontech) is used.

25 The preferred embodiment of the method of the invention wherein an inducible system is used advantageously allows to identify direct target genes controlled by the transcriptional factor, at any time during the plant development.

30 The creation of phenocopies by means of a chimeric DNA construct of the invention presents several other advantages :

The high penetrance (80 %) and dominance of phenocopies in primary transformants distinguishes this method from lower frequency antisense and cosuppression approaches, where a few individual progeny are selected.

The below examples and figures illustrate the invention without limiting its scope in any way.

LEGENDS OF FIGURES

Figure 1 represents a top view into the shoot apical meristem of a young wild-type *Arabidopsis* seedling. In addition to both cotyledons, it has developed four elaborated leaves plus two additional leaf primordia in the center covering the functional shoot apical meristem.

Figure 2 represents a top view into the shoot apical meristem of *Arabidopsis* phenocopy eng-STM of the same age as in figure 1. This phenocopy has two horizontal cotyledones and a single leaf in vertical position. A functional shoot apical meristem is missing and has presumably been consumed by initiation of the single leaf primordium.

Figure 3 represents a flower of wild-type *Arabidopsis thaliana* with outermost sepals (green), white petals, yellow stamen and central carpel.

Figure 4 represents a flower of *Arabidopsis thaliana* phenocopy eng-AP3. A single sepal has been removed frontally, two neighbouring normal sepals are slightly displaced left and right to allow insight into the second and third floral whorls. Petals and stamen are obviously replaced by sepaloid organs close to the central carpel and small filamentous structures, one visible at the bottom between the two sepaloid petals.

Figure 5 represents a eng-STM construct.

Figure 6 represents a eng-AP3 construct.

EXAMPLES

Example 1 : Construct eng-STM

Creation of pRT Ω eng

The cDNA clone that is used is D₂B clone as described in Poole et al, 1985. This clone was obtained by inserting a 2 kb EcoRI fragment of the engrailed cDNA in a pEMBL vector. The engrailed cDNA sequence is identical to the data base entry (Genbank Access. M 10017) except for the lack of the 3'

end downstream the EcoRI site gaattc, i.e the lack of the 3' end from nucleotide 2014 included.

From the cDNA clone D₂B clone a 929 Bp AflIII-BamHI fragment was isolated and inserted into NotI/BamHI cleaved pRTΩNot-Asc vector. Compatibility of the NotI/AflIII sites was achieved by fill in reaction with Klenow enzyme. The cDNA fragment covers the natural translation start and 298 amino acid residues of the engrailed protein. The BamHI site or a following XbaI site can be used to create translational fusions to proteins of interest.

The construct obtained was pRT Ω eng.

The pRTΩNot-Asc is described in Überlacker et al. (1996). It is a derivative of pRT100 (Töpfer et al., 1987) and contains the CaMV 35S promoter and a polyA signal of Cabb B.

ENG-STM cloning strategy

The sequence of the *STM* coding region that is used is disclosed in Long et al, 1996 and is identical to the database entry (Genbank – Access U32344).

To fuse the *STM* coding region to the engrailed repressor domain the frame of the unique XbaI cloning site in pRT Ω eng had to be shifted which was achieved by inserting a GATCTCGA adaptor into the upstream BamHI site, which was destroyed. The *STM* coding region previously amplified by reverse transcriptase PCR from Arabidopsis RNA with an 5' terminal XbaI (upstream the translation start ATG) was inserted into the XbaI site of the adapted pRT Ω eng. The *STM* coding sequences used contain a BamHI site immediately preceding the natural translation stop codon, which was used subsequently to create the c-terminal GR fusion.

ENG-STM without homeodomain (HD)

The homeodomain of STM was removed in an independent experiment. For that purpose, the plasmid pRT Ω eng-STM was first partially digested with HindII, secondly to completion with BamHI and ends were ligated after fill in reaction with Klenow enzyme. Thus, the construction eng-STM (Δ HD) was obtained.

Example 2 : Construct eng-AP3

ENG-AP3 cloning strategy

The sequence of the AP3 coding region that is used is disclosed in Jack et al., (1992) and is identical to the data base entry (Genbank – Access – D21125).

The AP3 cDNA clone coding region is fused to a myc epitope at its carboxy-terminus. The protein coding region including the myc epitope was amplified by PCR with primers adding terminal BamHI sites. The resulting BamHI fragment was inserted into the BamHI site of pRT Ω eng. This construct was designated as pRT Ω eng-AP3.

Example 3 : Construct eng-STM-GR

ENG-STM-GR

For fusion of the chimeric eng-STM polypeptide with the GR domain (Lloyd et al, 1994) a XhoI site in front of the TMV Ω leader was converted into a BamHI site. The eng-STM coding region was then inserted into pBI- Δ GR (Simon et al., 1996 ; Schena et al., 1991) as a BamHI fragment. The artificial BamHI site (see above) in front of the natural STM STOP codon fuses the eng-STM polypeptide in frame with the GR domain.

Example 4 : Obtention of transgenic plants

A – *Arabidopsis thaliana*

Both constructs, ENG-STM and ENG-AP3, were inserted into pGPTV-Bar Asc (Überlacker et al., 1996) by use of the AscI sites flanking the expression cassette and subsequently transferred into *Agrobacterium tumefaciens*, GV3101. Infiltration of *Arabidopsis* immature inflorescences followed the protocol of Bechtold et al., (1993) with minor modifications, as follows :

Concomitantly or after a short break axillary meristems are activated. The resulting secondary axes exhibit weaker phenotypic alterations and often develop quite normal rosettes. A peculiar feature of these axillary meristems is switching of meristem identity back and forth between vegetative and inflorescence state resulting in air-borne rosettes. The numbers of

inflorescence/vegetative cycles may vary between individual transgenic plants and exceptionally cycling may perpetuate to plant death. However, the majority of plants ultimately develop fertile flowers sometimes at terminal position. Other peculiarities are a reduced leaf size, alterations in leaf shape, fused leaves or fasciation of the inflorescence. To confirm heredity of the dominant-negative phenotype progeny of 20 T₁ plants exhibiting mild to strong *stm* phenocopies were analysed in the next generation. BASTA resistant T₂ progeny were obtained from 18 T₁ plants and phenocopies recovered in 16 families. The strength of phenotypic alterations and absolute numbers of affected progeny varied between different families, a variability presumably related to the expression level of the transgene, which may also account for the failure to recover phenocopies in the two residual families.

In contrast to *eng-STM* transgenic plants the phenotypic alterations caused by ectopic expression of the *eng-AP3* construct were exclusively restricted to the flower. In 70% of the primary transformants, petals were converted to sepals in the second floral whorl and filamentous structures replaced stamens in the third whorl (compare wild type in Figure 3 with transgenic plant in 4). Both homeotic transformations are reminiscent of strong *ap3* alleles and indicate that ectopic expression of the chimeric *eng-AP3* protein trans-dominantly copies the *ap3* loss of function phenotype. Due to the lack of functional stamen those *ap3*-phenocopy plants required backcrossing, but strictly segregating with the BASTA resistance marker this trans-dominant phenotype was transmitted to the next generation. The flowers from the plants transformed with the *eng-AP3* construct are male sterile.

In order to identify the chimeric *eng-AP3* protein in the cell, one could advantageously use the Myc epitope, which would be helpful to study for example whether the chimeric protein is expressed, whether it is cytoplasmatic or nuclear, whether it forms a heterodimer with the *pistilata* gene product or whether nuclear import is dependent on *pistilata*.

In summary the ectopic expression of both chimeric constructs, *eng-STM* and *eng-AP3*, results in stable, inheritable trans-dominant functions phenocopying *stm* or *ap3* loss of function alleles, respectively. Excess of

chimeric repressor transcription factor fusions can efficiently displace the native gene products from target genes.

Although expressed from a constitutive promoter both chimeric repressors eng-STM and eng-AP3 are obviously recruited by either DNA sequence specificity, protein-protein interactions or both to their natural target genes which are repressed resulting in the phenocopy of the respective loss of function mutant phenotype.

B – Maize

Genetic transformation of maize, whatever the method (electroporation, Agrobacterium, microfibres, particle gun) generally involves the use of an undifferentiated cells in rapid divisions that can still regenerate into a complete plant. This type of cells constitutes the embryogenic callus (of type II) of maize. Such callus is obtained from immature embryos having the genotype HI II or A188 x B73 according to the method and the media described in Dennehey et al, 1994, and may be multiplied and maintained by successive prickings every two-weeks on the initiation medium.

Plants are regenerated from these callus by modifying the hormonal and osmotic equilibrium of the cells according to the method described by Vain et al, 1989.

These plants are then acclimated in greenhouse where they can be crossed or self-pollinated.

A method of genetic transformation leading to a stable integration of the modified genes in the genome of the plant is used. This method involves the use of a gene gun. The target cells are fragments of callus having a surface area of 10 to 20 mm². Four hours before bombarding, the fragments are laid in the center of a Petri dish containing a culture medium identical to the initiation medium, further containing 0.2 M of mannitol + 0.2 M of sorbitol (16 fragments per dish). Tissues are then bombarded as previously described. The dishes are then sealed using Scellofrais® and cultivated in dark at 27°C. The first planting is effected 24 hours afterwards, then every two-weeks during three months in a medium identical to the initiation medium but containing a selective agent. The

selective agents may be for example active ingredients of herbicidal agents (Basta®, Round up®) or antibiotics (hygromycin, kanamycin).

After three months or sometimes sooner, callus that have not been inhibited by the selected agents, develop. These are usually composed of cells resulting from the division of a cell having integrated in its genome one or more copies of gene of interest. The frequency of obtaining such callus is about 0.8 callus per bombarded dish. Callus are identified, individualized, amplified and cultivated so as to regenerate plants. In order to avoid any interference with non transformed cells, all these steps are effected in culture media that contain the selective agent. The regenerated plants are acclimated and cultivated in the greenhouse where they can be crossed or self-pollinated.

In an other embodiment, the transformation of plants may be carried out with *Agrobacterium tumefaciens* and immature embryos, as described by Ishida et al., (1996).

Example 5 : Expression of the endogenous STM and AP3 genes

One explanation for these phenocopies might be homology dependent cosuppression mediated by high RNA levels of the transgene. To exclude this, transgenic eng-STM or eng-AP3 phenocopy plants were subjected to RNA gel blot and RT-PCR analysis. The chimeric transcripts derived from the CaMV 35S promoter were easily detectable in total or poly(A)⁺ RNA from pooled seedlings or flowers (12 individual progeny) probed with the STM or AP3 coding regions. Generally the high abundance of chimeric transcripts interfered with the detection of the shorter native mRNA in these gel blot experiments. Discrimination was achieved by probing with the natural 3'UTR sequences that are lacking in the transgenes. The result obtained for the STM 3'UTR confirms transcription of the native STM gene although at a significantly reduced level.

This reduction is not due to absence of the STM or AP3 transcripts in individual T₁ progeny, because the native transcripts could be detected by RT-PCR experiments in each individual phenocopy plant. To

Presence of the hormone in addition to the temporary arrest in SAM activity effects growth of leaf primordia. Petioles of immature leaves hardly elongated in comparison to control plant, the leaflets in *eng-STM-GR* plants consequently remain closely attached to the shoot axis. Also retarded is growth of the leaf lamina, which is often associated with leaf curling. Most of these defects in leaf development resemble observations in *stm* phenocopy plants, where initiated leaves generally grow slowly compared to wild-type plants or unaffected progeny. The only pronounced difference compared to *eng-STM* transgenic plants is the lack in petiole elongation, which, however, may be a consequence of the C-terminal fusion with the GR hormone-binding

domain. The dexamethasone inducibility strongly favours the assumption that the chimeric *eng-STM* protein exerts a dominant-negative function internally the plant cell nucleus.

5 **Example 7 : Expression of the C-terminal deletion polypeptide *eng-STM* (Δ HD)**

To further investigate the mechanism transgenic plants were raised expressing the C-terminal deletion polypeptide *eng-STM* (Δ HD). This deletion protein still spans 296 aa of *STM* protein sequence including the
10 conserved KNOX (Bürglin, 1997) and ELK domains (Kerstetter et al., 1994) but lacks the DNA-binding homeodomain. Transcription of the *eng-STM* (Δ HD) transgene was confirmed in BASTA resistant progeny by RT-PCR resulting in the predicted 192 bp amplicon (compared to 374 bp for *eng-STM*). The obtained primary transformants exhibited *stm* phenocopies in frequency and
15 strength indistinguishable from those observed with the full-length protein. With this construction, the trans-dominant negative function provided by the chimeric *eng-STM* protein is therefore independent of the DNA-binding activity contributed by the *STM* homeodomain.

Further results obtained in transgenic *Arabidopsis* indicate that
20 only 50 amino acids residues of the *STM* protein are sufficient to mediate *stm* phenocopies in fusion with the *eng* N-terminus.

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CLAIMS

1. A chimeric DNA construct comprising at least one
5 repressor sequence in transcriptional fusion with at least one plant-specific
sequence that codes for a protein or fragment thereof that binds to DNA or that
activates transcription either by binding to DNA itself or by interacting with a
DNA-binding protein ;
said repressor sequence being operably linked to elements allowing the
10 transcription of said fused sequences.

2. A chimeric DNA construct according to claim 1, wherein
said repressor sequence is at least the repressor domain of the *Drosophila*
engrailed gene (*eng*).

3. A chimeric DNA construct according to claim 1, wherein
said repressor sequence encodes for at least the Kruppel-associated box-A
(KRAB-A) domain of zinc finger proteins, or for at least the RE-1-silencing
transcription factor (REST), or for at least the BTB (for Broad-complex Tramtrac
20 and Bric) domain, also known as POZ-domain.

4. A chimeric DNA construct according to any of claims 1 to 3,
wherein said plant-specific sequence codes for at least a DNA-binding domain
of a plant transcription factor.

5. A chimeric DNA construct according to claim 4, wherein
said transcription factor is selected from the group consisting of STM, AP3,
ZmHox, Ms-41-A and Zm41-A.

6. A chimeric DNA construct according to any of claims 1 to 3,
wherein said plant-specific sequence activates transcription by interacting with
a DNA-binding protein.

7. A chimeric DNA construct according to any of claims 1 to 6, further comprising a sequence that encodes at least the hormone-binding domain of a steroid hormone receptor, said sequence being in frame with the fusion construct consisting of at least one repressor sequence in transcriptional fusion with at least one plant-specific sequence that codes for a protein or fragment thereof that activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein ;

whereby the nuclear localization of the chimeric protein, that is the product of the translation of said fusion construct is dependent on the application of said steroid hormone or analogues thereof.

8. A chimeric DNA construct according to claim 7, wherein said hormone-binding domain of steroid hormone receptor is the hormone-binding domain of a glucocorticoid receptor.

9. A chimeric DNA construct according to any of claims 1 to 8, wherein the elements allowing the transcription of said repressor sequence comprise a constitutive promoter.

10. A host cell transformed with a DNA construct according to any of claim 1 to 9.

11. A transgenic plant or parts thereof, said plant being transformed with a DNA construct according to any of claims 1 to 9, or deriving from a plant initially transformed with a DNA construct according to any of claims 1 to 9.

12. A method for obtaining a transgenic plant according to any of claims 10 or 11, wherein a DNA construct according to any of claims 1 to 9 is transferred and expressed in a plant cell and said cell is cultured under conditions for regenerating a whole transgenic plant.

13. Use of a chimeric DNA construct according to any of claims 1 to 9 for inhibiting the expression of a target gene in the genome of a plant, the transcription of which is activated by a protein encoded by a plant-specific sequence as defined in any of claims 1 to 9.

14. A method for determining the function of a transcription factor in plants, comprising the steps of:

i) fusing a sequence encoding said transcription factor to a repressor sequence to form a DNA construct as defined in any of claims 4 to 9 ;

ii) transforming plant cells with said DNA construct;

iii) culturing the plants obtained from the transformed cells and observing a phenocopy of a mutation correlated with the loss of expression of genes controlled by said transcription factor.

15. A method for identifying new genes in plants, comprising the steps of :

i) obtaining transgenic plants transformed with a chimeric DNA construct according to any of claims 1 to 6 ;

ii) comparing the RNA population from said transgenic plants with the RNA population of a plant that has not been transformed with a chimeric DNA construct according to any of claims 1 to 6, by amplifying the RNAs repressed by expression of the chimeric DNA construct, identified as genes inactive in said transgenic plants obtained in step i) but active in the plant that has not been transformed with a chimeric DNA construct.

16. A method for identifying new genes in plants, comprising the steps of :

i) obtaining transgenic plants transformed with a chimeric DNA construct according to claims 7 or 8, comprising a sequence that encodes at least the hormone-binding domain of a steroid hormone receptor, and/or according to claim 9, comprising an inducible promoter ;

ii) submitting said transgenic plants to an induction by means of a steroid hormone or analogues thereof and/or promoter inducer, whereby a phenocopy is created due the loss of expression of target genes ;

5 iii) comparing the RNA populations from said transgenic plants before and shortly after induction, by amplifying the RNAs repressed by expression of the chimeric DNA construct, identified as genes active before but inactive after induction.

PCT

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(54) Title: A METHOD FOR INHIBITING THE EXPRESSION OF TARGET GENES IN PLANTS		
(57) Abstract <p>This invention concerns a method for inhibiting the expression of target genes in plants wherein a chimeric DNA construct comprising at least one repressor sequence in transcriptional fusion with at least one plant-specific sequence that codes for a protein or fragment thereof that bind to DNA or that activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein.</p>		

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION				
COUNTRY	APPLICATION NO.	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
Europe	99420030.1	09/02/99		<input type="checkbox"/> YES <input type="checkbox"/> NO
	PCT/EP00/01524	09/02/00		<input type="checkbox"/> YES <input type="checkbox"/> NO
				<input type="checkbox"/> YES <input type="checkbox"/> NO
ALL FOREIGN APPLICATION(S), IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION				
				<input type="checkbox"/> YES <input type="checkbox"/> NO
				<input type="checkbox"/> YES <input type="checkbox"/> NO
				<input type="checkbox"/> YES <input type="checkbox"/> NO

Claim for Benefit of Prior U.S. Provisional Application(s)

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Provisional Application Number	Filing Date

Claim for Benefit of Earlier U.S./PCT Application(s) under 35 U.S.C. 120

(complete this part only if this is a divisional, continuation or C-I-P application)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

Power of Attorney

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